PB86-175502

Evaluation of Mutagenic and Carcinogenic Properties of Brominated and Chlorinated Acetonitriles: By-Products of Chlorination

(U.S.) Health Effects Research Lab.
Cincinnati, OH

1985

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1. REPORT NO. EPA/600/J-85/355	2.
3. R PB86-175502	
4. TITLE AND SUBTITLE EVALUATION OF MUTAGENIC AND CARCINOGENIC PROPERTIES OF BROMINATED AND CHLORINATED ACETONITRILES: By-Products of Chlorination (Journal Version)	5. REPORT DATE 1985
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) R. J. Bull, J. R. Meier, M. Robinson, H. P. Ringhand, R. D. Laurie, and J. A. Stober	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Toxicology and Microbiology Division, HERL/ORD/USEPA, Cincinnati, OH 45268	10. PROGRAM ELEMENT NO. ABMC1A
	11. CONTRACT/GRANT NO. Inhouse
12. SPONSORING AGENCY NAME AND ADDRESS Health Effects Research Laboratory (HERL) Office of Research and Development (ORD) U.S. Environmental Protection Agency (USEPA) Research Triangle Park, North Carolina 27711	13. TYPE OF REPORT AND PERIOD COVERED Journal
	14. SPONSORING AGENCY CODE EPA-600/11

15. SUPPLEMENTARY NOTES
P.O. Merrel Robinson, TOTB/TMD/HERL (CI) Citation: Fundamental and Applied Toxicology Vol. 5, No. 6 1065-1074 December 1985

16. ABSTRACT

The present study was undertaken to determine if chlorinated and brominated acetonitriles formed during the chlorination of drinking water possess mutagenic and/or carcinogenic properties. Chloroacetonitrile (CAN), dichloroacetonitrile (DCAN), trichloroacetonitrile (TCAN), bromochloroacetonitrile (BCAN), and dibromoacetonitrile (DBAN) were tested for their ability (1) to produce point mutations in the Salmonella/microsome assay, (2) to induce sister chromatid exchanges (SCE) in Chinese hamster ovary (CHO) cells in vitro, (3) to produce micronuclei in polychromatic erythrocytes in CD-1 mice, and (4) to act as tumor initiators in the skin of Sencar mice. DCAN and BCAN were found to be direct acting mutagens in Salmonella. All five haloacetonitriles induced SCEs in CHO cells in vitro. This activity paralleled the extent of chlorine substitution and was further enhanced in the dihaloacetonitrile series if bromine was substituted for chlorine. None of the haloacetonitriles showed evidence of activity in the mouse micronucleus assay. DBAN, BCAN, and CAN initiated tumors in the mouse skin with topical applications followed by a 20-week promotion schedule of TPA applications ($P < 0.02$). These data indicate that the haloacetonitriles do display mutagenic and carcinogenic properties in some test systems and the hazard associated with their occurrence in drinking water and production within the gastrointestinal tract require further evaluation.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

18. DISTRIBUTION STATEMENT Release to Public	19. SECURITY CLASS (This Report) Unclassified	21. NO. OF PAGES 11
	20. SECURITY CLASS (This page) Unclassified	22. PRICE

EPA/600/J-85/355
JOURNAL ARTICLE

FUNDAMENTAL AND APPLIED TOXICOLOGY 5, 1065–1074 (1985)

Evaluation of Mutagenic and Carcinogenic Properties of Brominated and Chlorinated Acetonitriles: By-products of Chlorination[1]

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Evaluation of Mutagenic and Carcinogenic Properties of Brominated and Chlorinated Acetonitriles: By-products of Chlorination. BULL, R. J., MEIER, J. R., ROBINSON, M., RINGHAND, H. P., LAURIE, R. D., AND STOBER, J. A. (1985) *Fundam. Appl. Toxicol.* 5, 1065–1074. The present study was undertaken to determine if chlorinated and brominated acetonitriles formed during the chlorination of drinking water possess mutagenic and/or carcinogenic properties. Chloroacetonitrile (CAN), dichloroacetonitrile (DCAN), trichloroacetonitrile (TCAN), bromochloroacetonitrile (BCAN), and dibromoacetonitrile (DBAN) were tested for their ability (1) to produce point mutations in the *Salmonella*/microsome assay, (2) to induce sister chromatid exchanges (SCE) in Chinese hamster ovary (CHO) cells *in vitro*, (3) to produce micronuclei in polychromatic erythrocytes in CD-1 mice, and (4) to act as tumor initiators in the skin of Sencar mice. DCAN and BCAN were found to be direct-acting mutagens in *Salmonella*. All five haloacetonitriles induced SCE in CHO cells *in vitro*. This activity paralleled the extent of chlorine substitution and was further enhanced in the dihaloacetonitrile series when bromine was substituted for chlorine. None of the haloacetonitriles showed evidence of activity in the mouse micronucleus assay. DBAN, BCAN, and CAN initiated tumors in the mouse skin with topical applications followed by a 20-week promotion schedule of 12-*O*-tetradecanoylphorbol-13-acetate applications ($p < 0.02$). These data indicate that the haloacetonitriles do display mutagenic and carcinogenic properties in some test systems and the hazard associated with their occurrence in drinking water and production within the gastrointestinal tract require further evaluation. © 1985 Society of Toxicology.

Chlorination of drinking water produces a variety of by-products (Jolley *et al.*, 1983). Most widely recognized are the trihalomethanes (Rook, 1974; Bellar *et al.*, 1974), but recently, the production of haloacetonitriles in certain water treatment plants was reported (Trehy and Bieber, 1981; Oliver, 1983). Although the substrate that reacts with chlorine in natural waters to produce haloacetonitriles has not been identified, proteinaceous material and certain amino acids react with chlorine to produce dichloroacetonitrile (Bieber and Trehy, 1983). Dihaloacetonitriles are also formed when chlorine is reacted with blue-green algae (Oliver, 1983) and with humic materials (Oliver, 1983; Coleman *et al.*, 1984). Furthermore, haloacetonitriles are formed in the gastrointestinal tract when rats are intubated with aqueous solutions of chlorine (Mink *et al.*, 1983).

Inclusion of bromide in aqueous solutions of chlorine results in the preferential substitution of bromine into organic substrates present in water (Coleman *et al.*, 1984) or in the gastrointestinal tract of rats (Mink *et al.*, 1983). In these reactions bromide is oxidized

[1] The research described in this article has been reviewed by the Health Effects Research Laboratory and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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0272-0590/85 $3.00

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by hypochlorite to form hypobromite (Farkas *et al.*, 1949).

Simmon *et al.* (1977) reported that dichloroacetonitrile was mutagenic in *Salmonella typhimurium*. The extent to which the other members of this chemical class share this property and whether they possess other genotoxic or carcinogenic activity were the objectives of the present study. These chemicals were examined for their ability to produce point mutations in *Salmonella*, to induce sister chromatid exchange (SCE) in Chinese hamster ovary cells (CHO), to induce micronuclei in polychromatic erythrocytes in the mouse, and to act as a tumor initiators in mouse skin.

METHODS

Chemicals. Chloroacetonitrile (CAN), dichloroacetonitrile (DCAN), trichloroacetonitrile (TCAN), and dibromoacetonitrile (DBAN) were obtained from Pfaltz and Bauer, Inc., Stamford, Connecticut. These compounds were purified by fractional vacuum distillation and analyzed for purity by gas chromatography. Their composition was confirmed by mass spectrometry. CAN and DCAN were both greater than 99% pure. TCAN was 98% pure (with 2% DCAN) and DBAN was 96% pure (with 4% tribromoacetonitrile). Bromochloroacetonitrile (BCAN) was synthesized in our laboratory by a modification of Wilt and Diebold's (1963) procedure for DBAN as suggested by Dr. T. J. Barber (Florida Atlantic University). Equimolar concentrations of *N*-chloro- and *N*-bromosuccinimide were premixed and added to a chilled aqueous solution of cyanoacetic acid. Following the removal of succinimide by filtration, the reaction mixture (filtrate) was extracted with methylene chloride, washed with 5% NaOH and water, and subsequently dried over anhydrous Na_2SO_4. The extract was purified by fractional vacuum distillation. The structure of the purified materials was confirmed by mass spectrometry. The final product was 93% pure (with 4% DBAN and 3% DCAN). 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) was obtained from Chemical Carcinogenesis, Eden Prairie, Minnesota.

Salmonella/microsome mutagenicity assay. Mutagenicity assays were conducted using the standard plate test method of Ames *et al.* (1975) with *S. typhimurium* strains TA1535, TA1537, TA1538, TA98, and TA100. Prior to use in assays, the presence of strain-specific markers were confirmed for each strain, including histidine dependence, crystal violet sensitivity, ampicillin resistance, sensitivity to ultraviolet light, spontaneous reversion rate, and response to positive control chemicals. A total of six to eight dose levels of the compounds were tested with a maximum dose which was based on the 50% cytotoxicity (LC50) level. Assays were performed with duplicate platings per dose and were conducted both with and without the addition of a liver S 9 fraction from Aroclor 1254-treated male Sprague–Dawley rats. The S 9 fraction and S 9 cofactor mix were prepared according to Ames *et al.* (1975). Positive and negative (solvent) controls were included with each assay. Dimethyl sulfoxide (Me_2SO) was used as the solvent. Cytotoxicity was determined by plating approximately two hundred TA100 cells on nutrient agar and checking for differential survival of treated vs untreated cells. LC50 values were determined by regression analysis of the kill curves after a log transformation of the data for percentage of cell killing. Mutagenic potencies were estimated using a least-squares regression analysis of the linear portion of the dose–response curves. A twofold above background dose-related response was the minimum requirement for determination of a positive mutagenic response.

Sister chromatid exchange with CHO cells. Chinese hamster ovary cells (CHO) were obtained from Dr. S. Wolff, University of California, San Francisco, and were cloned in the laboratory of Dr. A. Bloom, Columbia University, New York. This clone, designated WBL, has a modal chromosome number of 21 and an average cycle time of 12–14 hr. The cells were grown in McCoy's 5a medium supplemented with 10% fetal bovine serum, L-glutamine (1 mM), penicillin (1000 units/ml), and streptomycin (100 μg/ml). Cultures were initiated approximately 24 hr prior to treatment by seeding 1×10^6 cells per 75-cm^2 plastic flask in 10 ml medium. The highest doses of the chemicals tested were selected to ensure toxicity based upon results in another cell line (i.e., CCRF-CEM cells). Lower doses were tested in half-log increments by dilution of the highest dose. The concentration of diluent (Me_2SO) was maintained constant at 1% (v/v) of the medium. For assays without metabolic activation, cells were treated with the test compounds for 2 hr; then 5-bromo-2-deoxyuridine (BrdU) was added to the medium at a final concentration of 10 μM and the incubation continued for between 26 and 32 hr (depending on the percentage of cells in metaphase 2). For assays with metabolic activation, the cells were treated for 2 hr with the test compound in growth medium lacking fetal bovine serum, but containing an S 9 reaction mixture consisting of 2.4 mg NADP, 4.5 mg isocitric acid, and 15 μl S 9 liver homogenate fraction per milliliter of culture medium. After the 2-hr exposure, the cells were washed with phosphate-buffered saline and normal growth medium containing 10% fetal bovine serum, and 10 μM BrdU was added. Incubation was then continued for 26 hr longer. For both assay conditions (with and without S 9), colcemid (0.1 μg/ml final concn) was added for the last 2 hr of incubation. Metaphase cells were collected by mitotic shake off, then swollen with 0.075 M KCl, fixed in methanol:glacial acetic acid (3:1), and stained by a modified fluorescent plus Giemsa technique based on the methods of Perry and Wolff (1974) and Goto *et al.* (1978). All cultures were coded prior to fixation and the slides were scored blindly. Metaphase 2 cells were scored for the frequency of SCE per cell

and per chromosome. Fifty cells were scored per dose where cytotoxicity did not limit the availability of cells in metaphase. A one-tailed Student's *t* test ($\alpha = 0.01$) was used to test for a significant increase in the number of SCE per cell at each dose level over the concurrent controls. The significance level was adjusted for multiple comparisons as suggested by Bonferonni (Kotz and Johnson, 1981). The dose–response curves were used to determine the potencies of the chemicals for SCE induction by estimating the initial slopes by least-squares regression analysis.

In vivo mouse micronucleus assay. Adult strain CD-1 mice were purchased from Charles River Breeding Laboratories, Inc. Animals were randomly assigned to treatment groups. The haloacetonitriles were dissolved in 10% Emulphor and administered five times by oral gavage at dose levels of 12.5, 25, and 50 mg/kg/day on 5 consecutive days. The highest total dose level (i.e., 250 mg/kg) was chosen to approximate the single oral LD50, which ranges from 220 to 295 mg/kg for the five compounds (L. Condie, personal communication). Five males and five females were used per dose level. The vehicle control group received 10% Emulphor at the same volume (approximately 0.2 ml) as the treatment group. Six hours after the last administration, the animals were killed and bone marrow cells were collected from both tibiae. Slides were prepared from the cells and stained with May–Grunewald–Giemsa stain. Polychromatic erythrocytes (1000 per animal) were scored for the presence of micronuclei. All slides were coded prior to scoring and scorers were not aware of the animal's treatment group. A two-tailed Student's *t* test ($\alpha = 0.01$) was used to test for a significant increase in the frequency of micronuclei at each dose level over the negative control response. Data from male and female mice were analyzed both separately and combined.

Mouse skin bioassay. Female Sencar mice were obtained from Dr. T. Slaga of Oak Ridge National Laboratories, Oak Ridge, Tennessee (Experiments 1 and 2) and from Harlan Sprague–Dawley, Inc., Indianapolis, Indiana (Experiment 3), for use in the mouse skin initiation/promotion experiments. Experiment 3 was conducted to confirm the reduced activity at the high dose level of DBAN and TCAN. The haloganated acetonitriles were applied at doses of 200, 400, and 800 mg/kg (or 4, 8, and 16 mg/mouse) in 0.2 ml acetone to the shaved back. Six doses were applied over a 2-week period for total doses of 1200, 2400, and 4800 mg/kg (or 24, 48, and 96 mg/mouse). There were 40 animals on each exposure group. Two weeks after the last dose of the haloacetonitriles a promotion schedule involving the application of 1.0 μg of TPA in 0.2 ml acetone three times weekly was begun and continued for 20 weeks. Complete carcinogenic activity of the haloacetonitriles was assessed in a separate experiment where 800 mg/kg of CAN, DCAN, TCAN, and BCAN and 400 mg/kg DBAN was applied three times weekly for 24 weeks and animals observed for papilloma development. Experiments were also conducted utilizing the oral route of exposure. In this case, doses were limited to a maximum dose of 50 mg/kg administered six times over a 2-week period to avoid the acute toxic effects of these chemicals. Further conduct of these experiments was identical to the initiation/promotion experiments indicated above. The appearance and regression of tumors were charted weekly. Papillomas were included in the tumor count only if they were observed for three consecutive weeks at the same location. For each compound tested, the count of the total tumors and data on time to first tumor occurrence were analyzed for dose–response with a trend test based on Cox's regression model (Cox, 1972; Tarone, 1975).

Animals from the initiation/promotion experiments were maintained for one year to determine the incidence of squamous cell carcinomas resulting from the exposure to haloacetonitrile compounds. At necropsy all gross lesions were recorded and tissues with lesions were fixed in 10% buffered formalin for subsequent histopathological evaluation. The data for each compound and dose group were analyzed for a significant increase over the respective control response using Fisher's Exact Test.

RESULTS

Prior to testing the haloacetonitriles for mutagenicity in the Ames test, the cytotoxicity of the compounds to *S. typhimurium* strain TA100 was determined. The LC50 values (in micromoles per plate) were found to be 142.7 for CAN, 12.3 for DCAN, 4.5 for TCAN, 0.29 for BCAN, and 0.35 for DBAN. Because of this wide variation in the cytotoxicity of the chemicals, the dose ranges for testing the chemicals for mutagenicity also differed greatly. The mutagenicity test data are provided in Table 1. Test results with TA1537 and TA1538 were negative for all compounds tested (data not shown). CAN, DBAN, and TCAN also failed to produce dose-related mutagenic responses for strains TA1535, TA98, and TA100.

DCAN produced up to a 40-fold increase in the number of histidine revertants in *Salmonella* strain TA1535, more than a 6-fold increase in strain TA100, and a 2.5-fold increase in TA98 in the absence of S 9. Inclusion of S 9 in the assay system did not substantially alter the number of histidine revertants produced.

BCAN produced mutagenic resonses in *Salmonella* strains TA1535 and TA100. With TA1535 BCAN produced an increase in the number of histidine revertants over the con-

TABLE 1

MUTAGENICITY OF HALOACETONITRILES IN THE *Salmonella*/MICROSOME ASSAY

Compound	Dose (μmol/plate)	Total Rev.[a] revertants/plate[a]					
		TA1535		TA98		TA100	
		−S 9	+S 9	−S 9	+S 9	−S 9	+S 9
CAN	0.0	13	13	58	66	137	138
	5.0	14	11	55	66	132	143
	10.0	16	13	54	43	121	133
	20.0	8	11	64	54	90	117
	40.0	5	9	27	51	2	19
	80.0	0	1	0	7	0	0
	160.0	0	0	0	0	0	0
DCAN	0.0	10	12	15	21	92	115
	0.1	22	16	16	26	90	87
	0.2	29	21	15	31	111	97
	0.4	34	36	13	28	141	148
	0.8	46	44	18	31	164	155
	1.6	108	97	20	19	205	253
	3.1	136	166	23	26	324	303
	6.2	246	265	32	33	425	400
	12.4	371	352	48	52	598	498
TCAN	0.0	13	13	59	66	137	103
	0.4	15	8	62	66	153	129
	0.7	11	9	57	67	152	137
	1.5	12	11	65	63	146	137
	2.9	9	8	71	64	162	133
	5.9	13	10	59	62	144	151
	11.7	9	10	65	64	77	115
BCAN	0.9	23	18	36	46	175	140
	0.09	29	—	33	63	232	265
	0.17	38	—	42	60	216	286
	0.34	60	46	32	50	221	245
	0.68	21	81	37	47	201	341
	1.36	8	143	19	30	0	133
	2.72	0	96	0	0	0	0
	5.44	0	0	0	0	0	0
DBAN	0.0	26	18	32	33	122	126
	0.01	13	14	26	37	136	104
	0.03	13	14	20	39	128	97
	0.06	12	9	26	35	124	120
	0.12	14	17	15	39	128	102
	0.29	11	12	25	42	83	121
	0.58	1	26	1	40	0	74
2-NF[b]	10 μg			777 ± 216		1059 ± 101	
NaN_3	10 μg	840 ± 321					
2-AA	2.5 μg		241 ± 19		1047 ± 322		1366 ± 184

[a] Data are $\bar{X}$ values from duplicate plates for test compounds and $\bar{X}$ values ± standard deviation from four plates for positive control chemicals.

[b] 2-NF = 2-nitrofluorene; NaN_3 = sodium azide; 2-AA = 2-aminoanthracene.

centration range of 0 to 0.34 μmol/plate in the absence of S 9, but the response fell rapidly at higher concentrations due to toxicity, which was evident by the clearing of the background lawn. In the presence of S 9 the number of histidine revertants continued to increase linearly with a concentration up to 1.35 μmol/plate. The slopes of the linear portions of the dose–response curves were estimated to be 93 revertants/μmol with S 9 and 108 revertants/μmol without S 9. Therefore, the difference in the response range for mutagenic activity in the presence vs absence of S 9 appears to be due to a reduction in cytotoxicity by S 9, rather than to metabolic activation by S 9 to a more highly mutagenic form. This result may be explained by differential effects of S 9 on cytotoxic vs mutagenic lesions, similar to the effect of glutathione on the toxicity but not mutagenicity of enals (Marnett *et al.*, 1985). In strain TA100 BCAN did not produce a positive response in the absence of S 9 (based on a two-fold above background criteria), but did in the presence of S 9. However, the same general trend exists in the data from TA100 as was seen for TA1535; therefore, the effect of S 9 is probably not metabolic activation but rather reduction in cytotoxicity allowing detection of mutagenicity.

All of the haloacetonitriles were able to induce SCE in CHO cells *in vitro* (Table 2). Statistically significant increases in such activity were observable in the absence of S 9 mix with all compounds. In some cases, the maximal activity observed with the haloacetonitriles in the presence of S 9 exceeded that observed in the absence of S 9. However, this invariably involved higher concentrations of the chemical. The interpretation of this observation is complicated by the shorter treatment period that was necessary with S 9 present because of its cytotoxicity to CHO cells. Consequently, it should not be concluded that these data provide clear evidence that the haloacetonitriles require metabolic activation.

The potencies of the haloacetonitriles for SCE induction were calculated by least-squares regression analysis of the data in Table 2. The values obtained from assays that did not include S 9 (as SCE induced per millimole) were 50 ± 11 for CAN, 103 ± 26 for DCAN, 230 ± 26 for TCAN, 396 ± 69 for BCAN, and 469 ± 17 for DBAN.

The haloacetonitriles were tested *in vivo* for their ability to produce chromosome damage or loss by examining micronuclei production in polychromatic erythrocytes in the mouse. No significant increases in micronuclei were observed with any of the haloacetonitriles tested at doses ranging up to 50 mg/kg per day for 5 consecutive days (data not shown). The range of values for percentage of micronucleted cells was from 0.08 ± 0.03 to 0.37 ± 0.08 in the five control groups, and from 0.07 ± 0.03 to 0.38 ± 0.10 in the treated animals.

The haloacetonitriles were tested by two routes of administration as tumor initiators in mouse skin. Although there was a tendency in individual experiments involving oral exposures for increased papilloma yields with treatments by individual haloacetonitriles (data not shown), there were no statistically significant differences in the total number of tumors or in the time to first tumor when all experiments were combined for statistical analysis. The response to an oral dose of urethane (300 mg/kg), as a positive control, did produce an increase in papilloma yield consistent with past experience in our laboratory with the Sencar mouse (Bull *et al.*, 1984).

The haloacetonitriles were active as tumor initiators when applied topically to the shaved back of the Sencar mouse. In Fig. 1, the cumulative papilloma counts obtained from three experiments are summarized at 4 weeks following the end of TPA application. All of the haloacetonitriles produced increases in the average cumulative number of tumors per animal. In the case of DCAN and TCAN, these increases were not statistically significant ($p < 0.5$); somewhat greater and clearly dose-related increases in cumulative papilloma count were observed with CAN ($p = 0.02$) and BCAN ($p < 0.01$). DBAN produced significant ($p < 0.01$) increases in the cumulative tumor

TABLE 2

INDUCTION OF SISTER CHROMATID EXCHANGE IN CHINESE HAMSTER OVARY CELLS BY HALOACETONITRILES

		Assay without S-9 mix[a]		Assay with S-9 mix[b]	
Compound	Trial	μM concn in medium	SCE per cell ± SE	μM concn in medium	SCE per cell ± SE
CAN	1	0.0	8.3 ± 0.4	0.0	8.8 ± 0.4
		4.7	7.5 ± 0.4	15.8	8.8 ± 0.4
		15.8	8.7 ± 0.4	52.2	9.1 ± 0.4
		52.2	10.2 ± 0.4*	158.2	8.3 ± 0.4
		158.2	12.4 ± 0.5*	526.8	9.9 ± 0.4
	2	0.0	9.6 ± 0.4	0.0	9.4 ± 0.4
		79.1	13.5 ± 0.5*	237.3	10.5 ± 0.5
		118.7	15.8 ± 0.6*	474.6	11.7 ± 0.5*
		158.2	18.8 ± 0.6*	711.9	14.5 ± 0.5*
				949.2	16.6 ± 0.6*
DCAN	1	0.0	9.0 ± 0.4	0.0	8.9 ± 0.4
		0.2	8.9 ± 0.4	2.1	8.1 ± 0.4
		0.6	8.9 ± 0.4	6.2	8.8 ± 0.4
		2.1	8.1 ± 0.4	20.8	9.4 ± 0.4
		6.2	9.0 ± 0.4	62.2	13.2 ± 0.5*
		20.8	10.9 ± 0.5*	207.3	15.7 ± 0.6*
		62.2[c]		622.5[c]	
				0.0	7.5 ± 0.4
				124.5	9.4 ± 0.4
				155.6	9.8 ± 0.4
				186.8	11.7 ± 0.5*
				217.9	11.7 ± 0.5*
				249.0	12.5 ± 0.5*
TCAN	1	0.0	8.2 ± 0.4	0.0	10.1 ± 0.4
		0.6	7.9 ± 0.4	2.0	9.5 ± 0.4
		2.0	9.1 ± 0.4	6.6	8.8 ± 0.4
		6.6	8.7 ± 0.4	20.0	9.9 ± 0.4
		20.0	14.6 ± 0.4*	66.0	10.2 ± 0.5
	2	0.0	8.6 ± 0.4	0.0	8.9 ± 0.4
		15.0	11.4 ± 0.5*	50.0	10.5 ± 0.5
		20.0	14.4 ± 0.5*	75.0	11.7 ± 0.5*
		25.0	12.6 ± 0.5*	100.0	14.8 ± 0.8*
		30.0	15.7 ± 0.6*	125.0[c]	
		35.0	15.4 ± 0.5*		
BCAN	1	0.0	8.5 ± 0.4	0.0	8.4 ± 0.4
		2.1	9.6 ± 0.4	2.1	8.9 ± 0.4
		4.2	10.3 ± 0.4*	4.2	8.2 ± 0.4
		8.5	11.9 ± 0.5*	8.5	8.3 ± 0.4
		17.0[c]		17.0	9.8 ± 0.4
				34.0	15.2 ± 0.5*
	2	0.0	9.4 ± 0.4	0.0	9.5 ± 0.4
		6.5	12.2 ± 0.5*	27.2	10.3 ± 0.7
		8.7[c]		29.9	11.2 ± 0.5
				32.6	11.3 ± 0.5
				35.4	13.9 ± 0.5*
				38.1	19.1 ± 0.6*

TABLE 2—Continued

Compound	Trial	Assay without S-9 mix[a]		Assay with S-9 mix[b]	
		μM concn in medium	SCE per cell ± SE	μM concn in medium	SCE per cell ± SE
DBAN	1	0.0	7.7 ± 0.4	0.0	8.0 ± 0.4
		0.06	8.8 ± 0.4	0.17	8.8 ± 0.4
		0.17	10.6 ± 0.5*	0.58	8.8 ± 0.4
		0.58	9.7 ± 0.4*	1.73	8.9 ± 0.4
		1.73	10.2 ± 0.5*	5.78	9.1 ± 0.4
				17.33	11.6 ± 0.5*
	2	0.0	8.6 ± 0.4	0.0	8.9 ± 0.4
		1.7	8.8 ± 0.4	11.5	9.1 ± 0.4
		2.3	10.0 ± 0.4	17.3	9.3 ± 0.4
		2.9	10.3 ± 0.5	23.1	11.7 ± 0.5*
		3.5	10.5 ± 0.5*	28.9	13.5 ± 0.7*
		4.0	10.9 ± 0.5*	34.7	18.9 ± 0.9*

Note. Mitomycin C was used as a positive control for assays without S-9 at a level of 5 ng/ml medium. Cyclophosphamide was used as a positive control for assays with S-9 at a level of 0.5 μg/ml medium. Responses for mitomycin ranged from 23.7 ± 0.7 to 33.6 ± 0.8 SCE/cell, and responses for cyclophosphamide ranged from 20.5 ± 0.6 to 28.6 ± 0.8.

[a] Exposures to test chemicals varied from 28 to 35 hr depending on the extent of cell cycle delay observed.

[b] Exposure to test chemical was for 2 hr.

[c] Insufficient number of metaphase 2 cells present for scoring, due to toxicity.

* Significantly greater than concurrent solvent control at $p < 0.01$.

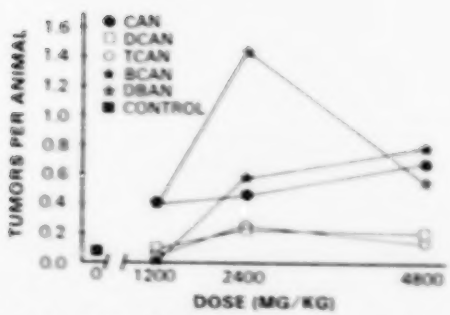

FIG. 1. Effects of haloacetonitriles on papilloma yield when applied topically as initiators in initiation/promotion studies in female Sencar mice. Following the administration of the initiating doses in six divided applications, TPA at 1 μg/mouse was applied three times weekly for 20 weeks. Results shown were taken 24 weeks after the start of TPA treatments and represent the combined results from three separate experiments, each experimental group involving 40 animals. Consequently, the control group includes a total of 120 animals started on experiment and all other points represent either 40 or 80 animals (see Table 3 for final numbers per experimental group).

yield that were progressive with total doses of 1200 and 2400 mg/kg, but a decreased tumor yield resulted at a dose of 4800 mg/kg. This dose–response pattern was confirmed in two separate experiments for both compounds at the two higher doses, although the data are combined for presentation in Figs. 1 and 2. High doses of DBAN proved to be quite irritating to mouse skin. Rather severe ulcerations on the shaved back of animals receiving the high dose of DBAN were observed and may have been responsible for the decreased response.

Figure 2 expresses data obtained from the topical applications of haloacetonitriles in terms of the percentage of animals that possessed one or more skin tumors during the first 24 weeks following the beginning of TPA application. These data reflect the same general pattern which was apparent when these data were expressed as tumors per animal.

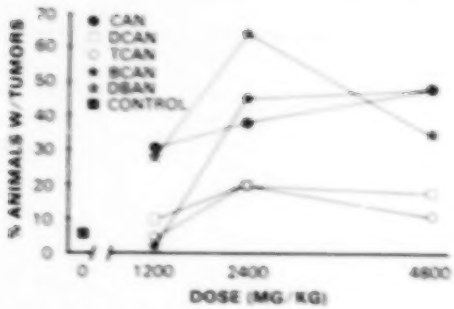

FIG. 2. Effect of haloacetonitrile administration on the percentage of tumor-bearing animals in each experimental group of female Sencar mice. Experimental conditions were as described in the legend for Fig. 1.

From both data sets, it is clear that DBAN is the more potent tumor initiator, followed by BCAN and CAN. TCAN and particularly DCAN were ineffective as tumor initiators in mouse skin. Tumors were not seen in animals receiving repeated applications of the haloacetonitriles at 800 mg/kg three times weekly for 24 weeks without TPA promotion (data not shown).

A tabulation of the histopathological classification of tumors present at autopsy of animals receiving topical applications of the haloacetonitriles is provided in Table 3. The differences in the tumor count presented in Table 3 and Fig. 2 are related to the regression of a number of the tumors that met criteria for the cumulative count used in Fig. 2 and/or to a loss of moribund animals to histopathological examination because of tissue autolysis or cannibalism. In general, however, the same relative pattern of tumor incidence was observed with chemical and dose at autopsy that was observed with cumulative papilloma counts after 24 weeks of promotion. In the case of DBAN, BCAN, and CAN the incidence of squamous cell carcinomas was increased significantly ($p < 0.05$) over that observed in the control groups. The only exception to this was that the high dose of CAN produced a larger proportion of squamous cell carcinomas than would have been predicted from the papilloma yield. As with the cumulative papilloma count, the yield of squamous cell carcinomas was decreased in two separate experiments when the total dose of DBAN was increased to 4800 mg/kg relative to the response observed at 2400 mg/kg. BCAN induced approximately the same yield of squamous cell carcinomas at 2400 and 4800 mg/kg (in 18 and 16% of the animals, respectively). It should also be noted that the significant increase in the tumor incidence produced by 2400 mg/kg TCAN was not reproduced in the third experiment.

DISCUSSION

The present work demonstrates that the haloacetonitriles most commonly produced in the chlorination of drinking water possess genotoxic activity. The ability of DCAN to produce point mutations in *Salmonella* confirms the earlier observation of Simmon *et al.* (1977) in strain TA100 and extends the results to include positive results in strains TA1535 and TA98. BCAN was the only other haloacetonitrile found capable of producing point mutations. BCAN was found to produce about 2.5 times as many revertants per micromole as DCAN in *Salmonella* strain TA1535 (108 vs 41). Positive results for both of the compounds in strain TA1535 indicates that they are capable of producing base-pair substitutions.

All five of the haloacetonitriles tested were capable of inducing SCE in CHO cells in the absence of S 9. Thus, all the haloacetonitriles appear to be direct-acting genotoxic agents. The order of potency of the haloacetonitriles in this system was DBAN > BCAN > TCAN > DCAN > CAN. Consequently, increasing halogen substitution results in a clear increase in the ability to induce SCE. Bromine substitution for chlorine in the dihaloacetonitrile series resulted in an additional fourfold increase in activity.

Testing of the haloacetonitriles in the micronucleus assay *in vivo* failed to provide any evidence of clastogenic activity in the intact animal. These results were obtained with administration of approximately 20% of the sin-

TABLE 3

HISTOPATHOLOGICAL DIAGNOSIS OF TUMORS RESULTING FROM TOPICAL TREATMENT WITH HALOGENATED ACETONITRILES IN THE SENCAR MOUSE

Treatment	Expt	Dose[a] (mg/kg)	N[b]	Number of animals with squamous cell tumors		Squamous cell tumors diagnosed		% Animals bearing carcinomas
				Number	%	Papilloma	Carcinoma	
CAN	1	1200	38	11	29**	7	4	10.5
	2	2400	37	11	30**	9	4	10.8
	2	4800	38	6	16	1	6	15.8*
DCAN	1	1200	39	4	10	4	0	0
	2	2400	35	4	11	2	3	8.6
	2	4800	35	1	3	1	0	0
TCAN	1	1200	34	2	6	1	1	2.9
	2	2400	36	11	31**	5	6	16.7*
	3	2400	38	1	3	0	1	2.6
	2	4800	36	3	8	2	1	2.8
	3	4800	29	2	7	1	1	3.4
BCAN	2	1200	35	1	3	0	1	2.9
	2	2400	37	7	19	0	7	18.9*
	2	4800	37	8	22*	3	6	16.2*
DBAN	1	1200	36	8	22*	6	2	5.6
	2	2400	35	17	49**	9	8	22.9**
	3	2400	35	16	46**	7	9	25.7**
	2	4800	37	7	19	5	2	5.4
	3	4800	37	3	11	1	2	7.4
Acetone	1	0.2 ml × 6	34	1	3	0	1	2.9
	2	0.2 ml × 6	37	3	8	3	0	0
	3	0.2 ml × 6	34	5	15	1	4	11.7

[a] Total doses of haloacetonitrile shown were delivered topically in six equal doses over a 2-week period. Then 2 weeks later animals were treated with 1.0 μg 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in 0.2 ml acetone topically 3 times weekly for 26 weeks.

[b] There were 40 animals initiated in each group. *N* = number available for histological examination.

* Significantly different from control, $p < 0.05$, Fisher exact test.

** Significantly different from control, $p < 0.01$, Fisher exact test.

gle oral LD50 of these chemicals for a period of 5 consecutive days. These chemicals have also failed to produce spermhead abnormalities in mice (Meier *et al.*, 1985). The reasons for the discordant results between the *in vitro* and *in vivo* genotoxicity assays are unknown but may be attributable to differences in assay endpoints, failure to reach the target issue, or *in vivo* detoxification mechanisms.

Experiments in the Sencar mouse demonstrated that the haloacetonitriles, applied topically, are capable of initiating tumors in mouse skin. The most potent derivative in terms of peak tumor yield tested was DBAN followed by BCAN and CAN. However, the dose–response characteristics with CAN seemed to indicate more potent action at lower doses. Unlike the results with SCE induction in CHO cells, there was no relationship between chlorine substitution and potency of the haloacetonitriles as tumor initiators. Bromine substitution did produce substantial increases

in the potency of the dihaloacetonitriles as tumor initiators (i.e., relative to DCAN). This latter result was consistent with the results obtained in CHO cells.

In conclusion, a number of the haloacetonitriles that are associated with drinking water treatment are capable of inducing point mutation in *Salmonella*, increasing the frequency of SCE in CHO cells *in vitro*, and initiating tumors in mice when applied topically to the skin. However, in every case the activity of these chemicals is low relative to other recognized carcinogenic and mutagenic compounds. In those instances where systemic administration was employed (micronucleus assay and skin tumor assays) the compounds appeared to be without significant activity. It can be inferred that they probably constitute a relatively low carcinogenic hazard to man. This question, however, does require further study.

ACKNOWLEDGMENTS

Portions of the mutagenicity testing were performed under Contract 68-03-2977 with Litton Bionetics, Inc., Kensington, Maryland. The authors express their appreciation to Diane Routledge and Pat Underwood for their assistance in the preparation of this manuscript.

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